Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 527 (2005) 23–30
www.elsevier.com/locate/ejphar

Inhibition of 1-methyl-4-phenylpyridinium-induced mitochondrial dysfunction and cell death in PC12 cells by sulfonylurea glibenclamide

Chung Soo Lee *, Yun Jeong Kim, Hyun Hee Ko, Eun Sook Han

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea

Received 26 May 2005; received in revised form 27 September 2005; accepted 7 October 2005
Available online 23 November 2005

Abstract

The present study investigates the effect of sulfonylurea glibenclamide on the cytotoxicity of 1-methyl-4-phenylpyridinium (MPP^+) in differentiated PC12 cells in relation to changes in the mitochondrial membrane permeability. Glibenclamide and tolbutamide reduced the MPP^+-induced cell death and GSH depletion concentration dependently with a maximal inhibitory effect at 5–10 μM. Despite the toxic effect at 20 μM, sulfonylureas showed an inhibitory effect. *N*-Acetylcysteine, superoxide dismutase, catalase, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride inhibited the cytotoxicity of MPP^+. Glibenclamide attenuated the nuclear damage, changes in the mitochondrial membrane permeability, caspase-3 activation and formation of reactive oxygen species due to MPP^+ in PC12 cells. The results show that glibenclamide may reduce the MPP^+-induced viability loss in PC12 cells by suppressing the changes in the mitochondrial membrane permeability, leading to the release of cytochrome *c* and subsequent activation of caspase-3, which are associated with the increased reactive oxygen species formation and depletion of GSH.
© 2005 Elsevier B.V. All rights reserved.

Keywords: 1-Methyl-4-phenylpyridinium; Glibenclamide; PC12 cell; Mitochondrial membrane permeability; Cell injury

1. Introduction

The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis (Crompton, 1999). Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome *c*, osmotic swelling and loss of oxidative phosphorylation. The permeability transition pore plays a critical role in cell death due to neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). Neuronal cell death due to MPP^+ is mediated by opening of the mitochondrial permeability transition pore, release of cytochrome *c* and activation of caspases (Cassarino et al., 1999; Lotharius et al., 1999; Lee et al., 2000, 2002). Infusion of MPP^+ into the brains of mice and rats increases the formations of lipid peroxides and hydroxyl radicals in the striatum (Rojas and Rios, 1993; Obata, 2003). In contrast, MPP^+ does not induce lipid peroxidation in PC12 cells and antioxidants do not prevent the MPP^+-induced decrease in [^{3}H] dopamine uptake in cells (Fonck and Baudry, 2001). It is therefore uncertain whether reactive oxygen species is involved in cytotoxicity of MPP^+.

Antidiabetic sulfonylureas, including glibenclamide, exhibit an inhibitory effect on surface and mitochondrial ATP-sensitive potassium (K_{ATP}) channels (Szewczyk and Marbán, 1999; Busija et al., 2004). Sulfonylureas induce the Ca^{2+} influx and insulin release in and from pancreatic islets, which is mediated by inhibition of K_{ATP} channels (Emilien et al., 1999). The K_{ATP} channels in various types of cells are suggested as a therapeutic target in the treatment of diseases, including diabetes, heart failure and tissue ischemia and as an action site for neuroprotection (Busija et al., 2004). Opening of surface K_{ATP} channels leads to hyperpolarization of cells and inhibition of Ca^{2+} influx (Szewczyk and Marbán, 1999; Busija et al., 2004). Meanwhile, the activation of the mitochondrial K_{ATP} channels, which are identified in the inner membrane of mitochondria, causes a mitochondrial depolarization. Diazoxide, a K^+ channel opener, has been shown to protect neuronal cells against the toxicities of amyloid β-peptide and glutamate, which is inhibited by 5-

* Corresponding author. Tel.: +82 2 820 5659; fax: +82 2 815 3856.
E-mail address: leecs@cau.ac.kr (C.S. Lee).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.10.008

hydroxydecanoate, a selective mitochondrial K_{ATP} channel inhibitor, and glibenclamide (Goodman and Mattson, 1996; Nagy et al., 2004).

Unlike the effect on diazoxide, the effect of sulfonylureas against neuronal cell injury has not been elucidated. Glibenclamide and tolbutamide are demonstrated to induce apoptotic cell death in cultured human islet β-cells by closing K_{ATP} channels (Rustenbeck et al., 2004; Maedler et al., 2005). Glibenclamide induces cell death in rat insulinoma cell line by increasing Ca^{2+} influx (Iwakura et al., 2000). In contrast, the toxic effect of tolbutamide on pancreatic islets is not mediated by Ca^{2+} influx (Rustenbeck et al., 2004). Nevertheless, sulfonylurea gliclazide reduces hydrogen peroxide-induced apoptotic cell death in pancreatic β-cells (Kimoto et al., 2003). In addition, the effect of sulfonylureas against toxic or oxidative neuronal cell injury is uncertain. The action mechanism also has not been clarified. The aim of the present study was to investigate the effect of glibenclamide on the cytotoxicity of MPP^+ in differentiated PC12 cells in relation to changes in the mitochondrial membrane permeability.

2. Materials and methods

2.1. Materials

TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD); Quantikine® M rat/mouse cytochrome *c* assay kit was from R&D systems (Minneapolis, MN); anti-cytochrome *c* (A-8) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); horseradish peroxidase-conjugated anti-mouse IgG was from EMD-Calbiochem. Co. (La Jolla, CA); SuperSignal® West Pico chemiluminescence substrate was from PIERCE Biotechnology Inc. (Rockford, IL); ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA); and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP) was from OXIS International Inc. (Portland, OR). Glibenclamide, tolbutamide, 1-methyl-4-phenylpyridinium (MPP^+), superoxide dismutase (SOD, from bovine erythrocytes; 2500–7000 units/mg protein), catalase (from bovine liver; 10,000–25,000 units/mg protein), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), phenylmethylsulfonylfluoride (PMSF) and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

2.2. Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, South Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin as described in the manual of the cell line bank. Cells were differentiated by treating with 100 ng/ml 7S nerve growth factor for 9 days (Tatton et al., 2002). Cells were washed with RPMI medium containing 1% FBS 24 h before experiments and replated onto the 96- and 24-well plates.

2.3. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C. The medium was incubated with 10 μl of 10 mg/ml MTT solution for 2 h. The culture medium was removed and 100 μl dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA). Cell viability was expressed as a percentage of the value in control cultures.

2.4. Morphological observation of nuclear change

PC12 cells (1×10^6 cells/ml) were treated with MPP^+ for 24 h at 37 °C and the nuclear morphological change was assessed using the Hoechst dye 33258 (Oberhammer et al., 1992). Cells were washed with 1 ml of phosphate-buffered saline (PBS) and incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature. Nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo, Japan).

2.5. Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. PC12 cells (1×10^5 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed with PBS and fixed with 3.7% buffered formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3′-ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT) and the nucleotide was detected using a streptavidin-horseradish peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

2.6. Flow cytometric measurement of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential during the MPP^+-induced apoptosis in PC12 cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ (Isenberg and Klaunig, 2000). Cells (1×10^6 cells/ml) were treated with H_2O_2 for 24 h at 37 °C, $DiOC_6(3)$ (40 nM) added to the medium and cells incubated for 15 min at 37 °C. After centrifugation at 412×*g* for 10 min, the supernatants were removed and the pellets suspended in 1 ml of

PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson, San Jose, CA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.7. Measurement of cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was assessed by using a solid-phase enzyme-linked immunosorbent assay kit and by Western blot analysis. PC12 cells (5×10^5 cells/ml for ELISA assay and 5×10^6 cells for Western blotting) were harvested by centrifugation at 412×*g* for 10 min, washed twice with PBS, resuspended in buffer (in mM): sucrose 250, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, dithiothreitol 0.5, PMSF 0.1 and HEPES–KOH 20, pH 7.5 and homogenized further by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at 100,000×*g* for 30 min and the supernatant was used for analysis of cytochrome *c*. The supernatants were added to the 96-well microplates coated with monoclonal antibody specific for rat/mouse cytochrome *c* that contains cytochrome *c* conjugate. The procedure was performed according to the manufacturer's instructions. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding diluted solutions of cytochrome *c* standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as ng/ml by reference to the standard curve.

For Western blotting, supernatants were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples (25 μg/ml protein) were loaded onto each lane of 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked for 2 h in TBS (50 mM Tris–HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat dried milk. The membranes were labeled with anti-cytochrome *c* (diluted 1:1000 in TBS containing 0.1% Tween 20) for 2 h at room temperature with gentle agitation. After four washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) for 2 h at room temperature. Protein bands were identified with the enhanced chemiluminescence detection using SuperSignal® West Pico chemiluminescence substrate.

2.8. Measurement of caspase-3 activity

PC12 cells (2×10^6 cells/ml) were treated with MPP^+ for 24 h at 37 °C and caspase-3 activity was determined according to the user's manual for the ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and incubated for 1 h at 37 °C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from the absorbances of *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM).

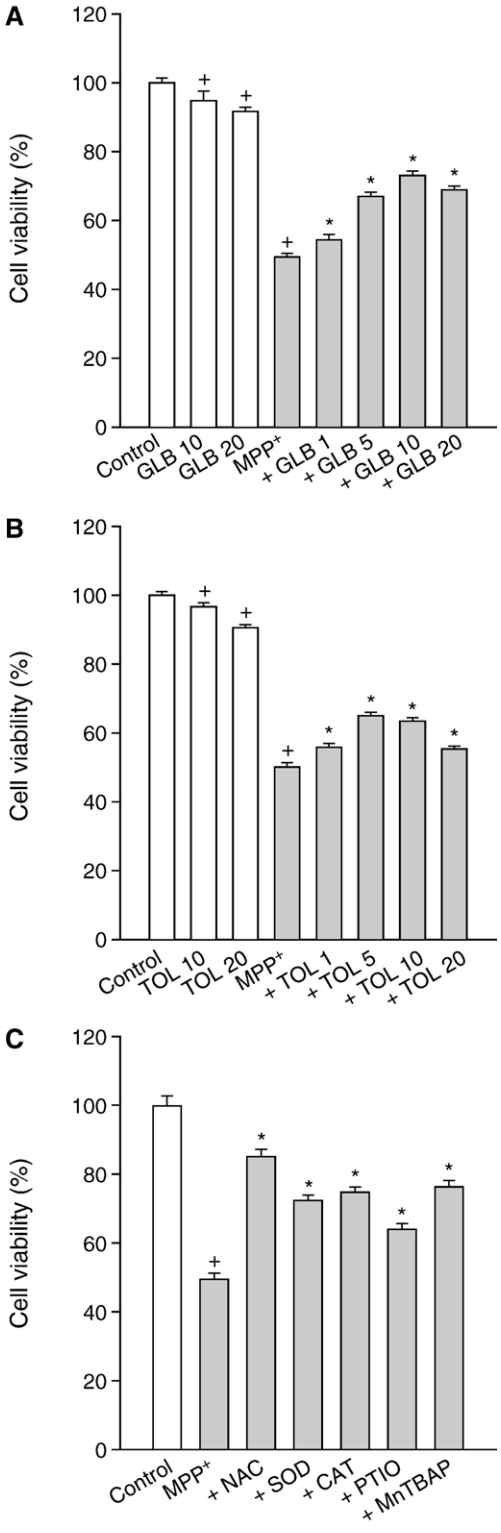

Fig. 1. Effect of glibenclamide on MPP^+-induced cell death. In experiments A and B, PC12 cells were pre-treated with glibenclamide or tolbutamide (GLB or TOL, 1–20 μM) for 30 min, exposed to 500 μM MPP^+ for 24 h, and cell viability was determined. In experiment C, cells were treated with 500 μM MPP^+ in the presence of the scavengers (1 mM *N*-acetylcysteine [NAC], 10 μg/ml SOD, 10 μg/ml catalase [CAT], 25 μM carboxy-PTIO [PTIO] and 30 μM Mn-TBAP [MnTBAP]). Data represent the mean±S.E.M. of six replicate values in two separate experiments. $^+P<0.05$ compared to control (percentage of control) and $*P<0.05$ compared to MPP^+ alone.

One unit of the enzyme was defined as the activity producing 1 nmol of *p*-nitroanilide.

2.9. Measurement of intracellular reactive oxygen species formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu et al., 1998). PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed, suspended in FBS-free RPMI, incubated with 50 μM dye for 30 min at 37 °C and washed with PBS. The cell suspensions were centrifuged at 412×*g* for 10 min and medium was removed. Cells were dissolved with 1% Triton X-100 and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

2.10. Measurement of total glutathione

The total glutathione (reduced form GSH+ oxidized form GSSG) was determined using glutathione reductase (van Klaveren et al., 1997). PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, centrifuged at 412×*g* for 10 min in a microplate centrifuge and the medium removed. The pellets were washed twice with PBS, dissolved with 2% 5-sulfosalicylic acid (100 μl) and incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37 °C. Glutathione reductase (20 μl, 100 units/ml) was added and the mixture incubated for a further 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples.

2.11. Statistical analysis

Data are expressed as the mean±S.E.M. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

3. Results

3.1. Inhibition of MPP^+-induced cell death and nuclear damage by glibenclamide

The effect of various concentrations of glibenclamide on the cytotoxicity of MPP^+ was assessed in PC12 cells that are differentiated by nerve growth factor. PC12 cells exposed to 500 μM MPP^+ for 24 h exhibited about 51% of cell viability loss. Glibenclamide significantly reduced the MPP^+-induced cell death concentration dependently with a maximum inhibition at 10 μM (48%); beyond this concentration, the inhibitory effect declined (Fig. 1A). Despite the cytotoxic effect at 20 μM, glibenclamide attenuated the cytotoxicity of MPP^+. Treatment with glibenclamide (20 μM) alone caused 8% cell viability loss. We examined whether antidiabetic sulfonylurea tolbutamide also shows a protective effect on the cytotoxicity of MPP^+. Similar to glibenclamide, the addition of tolbutamide significantly reduced the cytotoxic effect of MPP^+ on PC12 cells and, at 5 μM, it shows a maximal inhibitory effect (Fig. 1B). To assess the cytotoxic effect of tolbutamide itself, PC12 cells were treated with the compounds in the absence of MPP^+ for

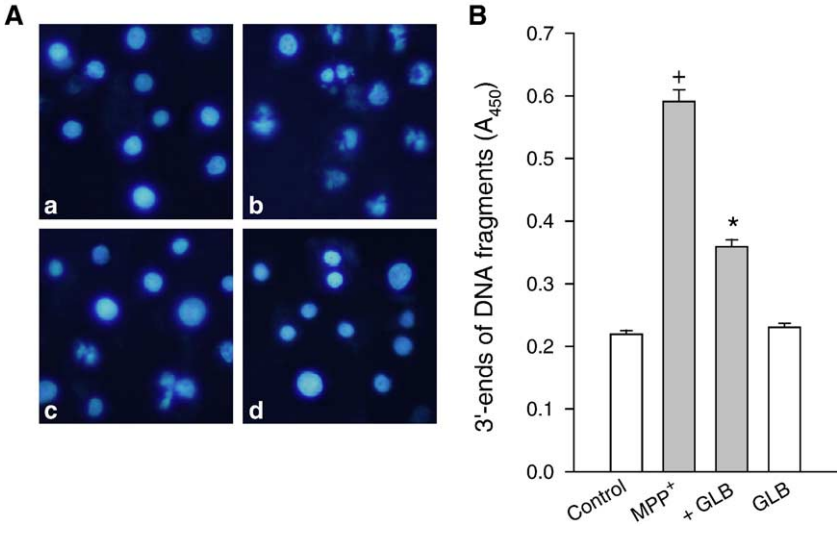

Fig. 2. Inhibition of MPP^+-induced nuclear damage by glibenclamide. PC12 cells were treated with 500 μM MPP^+ in the presence of 10 μM glibenclamide (GLB). In experiment A, cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with MPP^+ (b), cells treated with MPP^+ and 10 μM glibenclamide (c), and cells treated with glibenclamide (d). (a)–(d) are representatives of four different experiments. In experiment B, the 3′-ends of DNA fragments were detected as described in Materials and methods. Data are expressed as absorbance and represent the mean±S.E.M. of six replicate values in two separate experiments. $^+P<0.05$ compared to control and $*P<0.05$ compared to MPP^+ alone.

24 h. Although tolbutamide (20 μM) alone caused 9% cell viability loss, it had an inhibitory effect against the cytotoxicity of MPP^+.

We examined whether the toxic effect of MPP^+ against PC12 cells is mediated by the actions of reactive oxygen species and nitrogen species. Treatment with 1 mM thiol compound *N*-acetylcysteine, 10 μg/ml SOD (a superoxide scavenger), 10 μg/ml catalase (a scavenger of hydrogen peroxide), 25 μM carboxy-PTIO (a scavenger of nitric oxide) and 30 μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics SOD) reduced cell death caused by 500 μM MPP^+ (Fig. 1C).

To assess apoptotic cell death due to MPP^+ and clarify the inhibitory effect of glibenclamide on the cytotoxicity of MPP^+, we investigated the effect of glibenclamide on the nuclear morphological changes observed in the MPP^+-treated cells. Nuclear staining with Hoechst 33258 demonstrated that control PC12 cells had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were evident in cells treated with 500 μM MPP^+ (Fig. 2A). Glibenclamide (10 μM) decreased the MPP^+-induced nuclear damage, while the nuclear morphology in cells exposed to glibenclamide alone was similar to that in the control cells.

During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. Fragmented DNA was assessed by measuring the binding of dNTP to the 3′-ends of DNA fragments and detection by a quantitative colorimetric assay. PC12 cells were treated with 500 μM MPP^+ in the presence or absence of glibenclamide. Control cells showed absorbance of 0.219±0.006 (mean±S.E.M. of six experiments), while exposure to 500 μM MPP^+ for 24 h increased the absorbance about 2.7-fold (Fig. 2B). Glibenclamide (10 μM) significantly reduced the MPP^+-induced increase in absorbance, while absorbance in cells treated with glibenclamide alone was not significantly different from that in control cells.

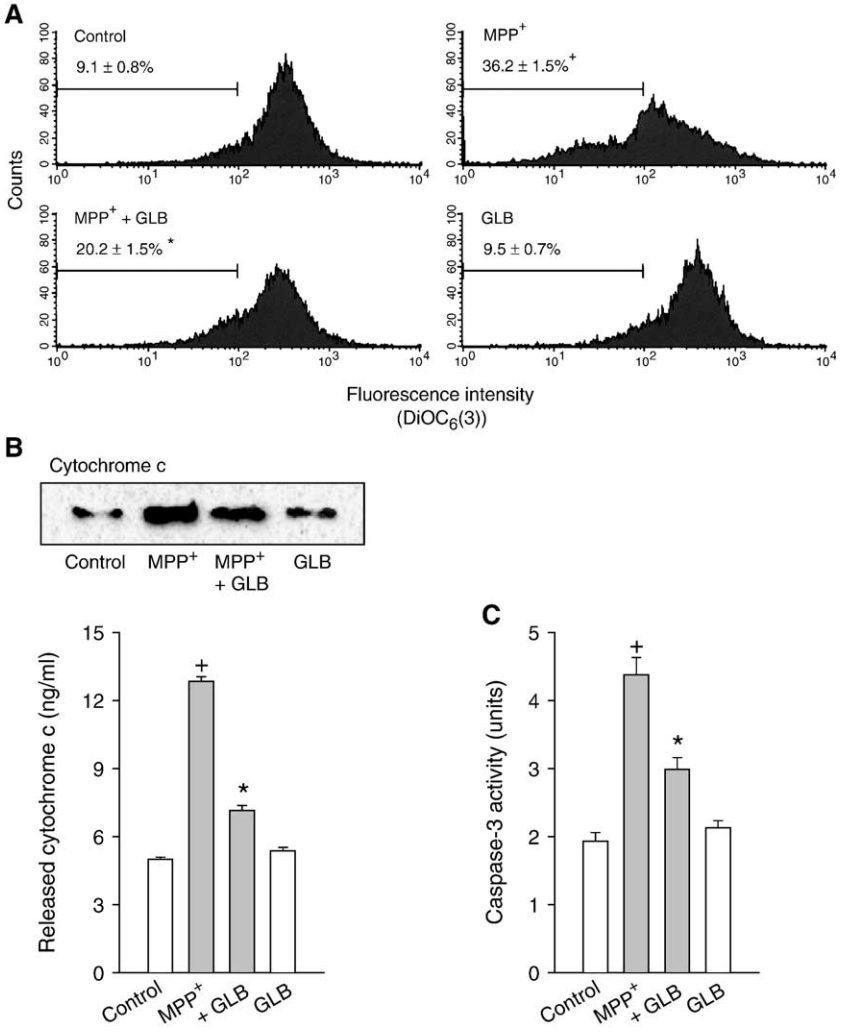

Fig. 3. Effect of glibenclamide on MPP^+-induced loss of the mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-3. PC12 cells were treated with 500 μM MPP^+ in the presence of 10 μM glibenclamide (GLB). Data are expressed as the percentage of cells with depolarized mitochondria for the mitochondrial membrane potential (A), ng/ml for cytochrome *c* release (B) and units for caspase-3 activity (C), and represent the mean±S.E.M. of three to six replicate values in two to three separate experiments. $^+P<0.05$ compared to control and $*P<0.05$ compared to MPP^+ alone. The levels of cytochrome *c* in the cytosolic fractions were analyzed by Western blotting with anti-cytochrome *c* antibody (B). Data are representative of three different experiments.

3.2. Effect of glibenclamide on MPP^+-induced changes in the mitochondrial membrane permeability

We assessed the cytotoxic effect of MPP^+ by investigating its effect on the mitochondrial membrane permeability. Changes in the mitochondrial transmembrane potential in PC12 cells treated with 500 μM MPP^+ were quantified by flow cytometry with the dye $DiOC_6(3)$. Exposure of PC12 cells to MPP^+ for 24 h increased the percentage of cells with depolarized mitochondria (characterized by low values of the transmembrane potential). Glibenclamide (10 μM) significantly inhibited the MPP^+-induced increase in cells with depolarized mitochondria, while the mitochondrial transmembrane potential in cells treated with glibenclamide alone was not significantly different from that in control (Fig. 3A).

The MPP^+-induced changes in the mitochondrial membrane permeability were assessed by measuring a release of cytochrome *c* into the cytosol and subsequent activation of caspase-3. PC12 cells treated with 500 μM MPP^+ showed a significant increase in the cytochrome *c* release and activation of caspase-3 activity. Glibenclamide (10 μM) significantly depressed the MPP^+-induced release of cytochrome *c* and increase in caspase-3 activity, while glibenclamide alone did not cause significantly the cytochrome *c* release and caspase-3 activation (Fig. 3B,C). The inhibitory effect of glibenclamide on the MPP^+-induced release of cytochrome *c* was also identified by Western blot analysis.

3.3. Effect of glibenclamide on MPP^+-induced formation of reactive oxygen species and depletion of GSH

To determine whether reactive oxygen species are involved in the MPP^+-induced cell death in PC12 cells, we investigated the formation of reactive oxygen species within cells by

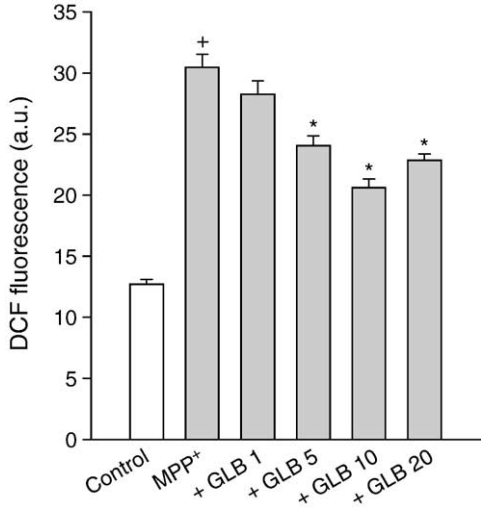

Fig. 4. Effect of glibenclamide on MPP^+-induced reactive oxygen species formation. PC12 cells were treated with 500 μM MPP^+ in the presence of glibenclamide (GLB, 1–20 μM). Data are expressed as arbitrary units of fluorescence and represent the mean±S.E.M. of six replicate values in two separate experiments. $^+P<0.05$ compared to control and $*P<0.05$ compared to MPP^+ alone.

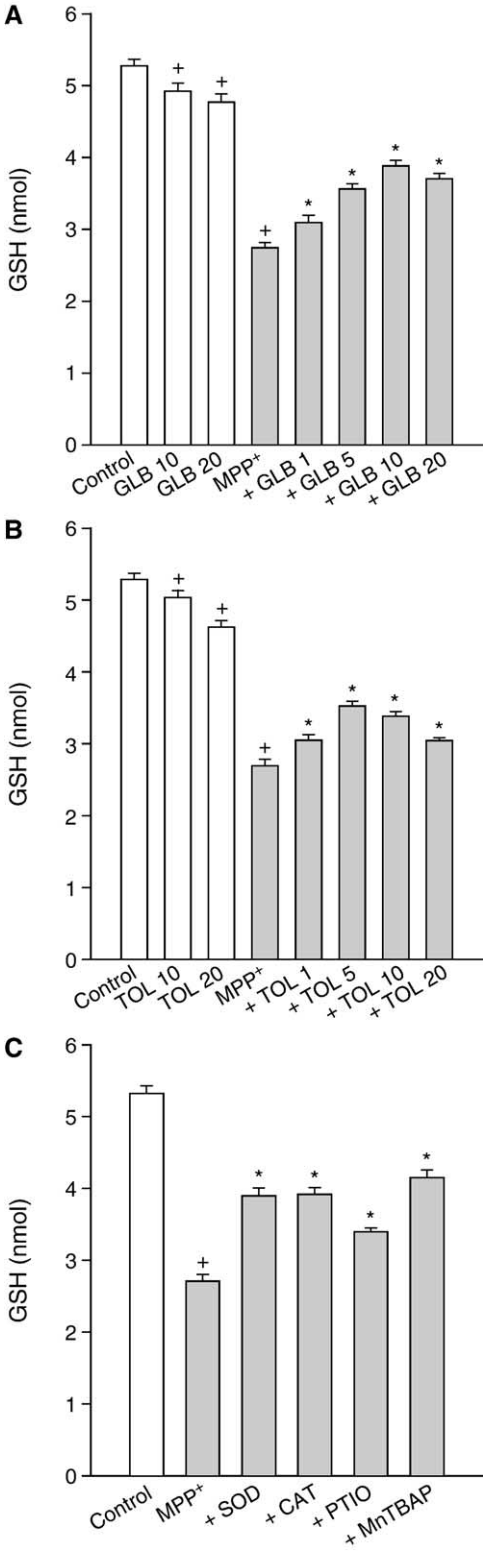

Fig. 5. Inhibition of MPP^+-induced depletion of GSH by glibenclamide. In experiments A and B, PC12 cells were treated with 500 μM MPP^+ in the presence of glibenclamide or tolbutamide (GLB or TOL, 1–20 μM). In experiment C, cells were treated with 500 μM MPP^+ in the presence of the scavengers (10 μg/ml SOD, 10 μg/ml catalase [CAT], 25 μM carboxy-PTIO [PTIO] and 30 μM Mn-TBAP [MnTBAP]). Data are expressed as nmol of GSH/mg protein and represent the mean±S.E.M. of six replicate values in two separate experiments. $^+P<0.05$ compared to control and $*P<0.05$ compared to MPP^+ alone.

monitoring a conversion of $DCFH_2$-DA to DCF. PC12 cells exposed to 500 μM MPP^+ showed a significant increase in DCF fluorescence. Glibenclamide (1–20 μM) inhibited the MPP^+-induced increase in DCF fluorescence and, at 10 μM, it showed a maximum inhibition (Fig. 4).

Drops in cellular GSH levels increase the sensitivity of neurons to the toxic effect of neurotoxins and are associated with mitochondrial dysfunction (Chandra et al., 2000). We investigated the effect of glibenclamide and tolbutamide on the MPP^+-induced decrease in GSH contents. The thiol content in the control PC12 cells was 5.27±0.09 nmol/mg protein. Treatment with 500 μM MPP^+ for 24 h depleted GSH contents by 48%. Glibenclamide and tolbutamide (1–10 μM) significantly inhibited the MPP^+-induced depletion of GSH and showed a maximum inhibition at 5–10 μM (Fig. 5A,B). Although glibenclamide and tolbutamide at 20 μM decreased the GSH contents, both compounds significantly attenuated the MPP^+-induced GSH depletion. The addition of antioxidant enzymes (10 μg/ml of SOD and catalase), 25 μM carboxy-PTIO and 30 μM Mn-TBAP inhibited the MPP^+-induced depletion of GSH in PC12 cells (Fig. 5C).

4. Discussion

Although rat PC12 cells are not brain dopaminergic neurons, these cells are able to produce dopamine and express dopamine transporter (Kadota et al., 1996). Upon nerve growth factor stimulation, PC12 cells not only display abundant neuritic growth, but also adopt a neurochemical dopaminergic phenotype. On the basis of the character of PC12 cells, we assessed the cytotoxicity of MPP^+ against dopaminergic neurons using the PC12 cells differentiated with nerve growth factor. The cytotoxic effect of MPP^+ on differentiated PC12 cells was determined by measuring cell viability, nuclear damage and activation of caspase-3. Formation of the mitochondrial permeability transition causes a release of cytochrome *c* from mitochondria and subsequent activation of caspase-3 that is involved in apoptotic cell death (Crompton, 1999). In this study, the MPP^+-induced apoptotic cell death in differentiated PC12 cells was demonstrated by the condensation and fragmentation of nuclei and by changes in the mitochondrial membrane permeability, leading to the cytochrome *c* release and caspase-3 activation.

The MPP^+ treatment elicits the respiratory chain inhibition, leading to the formation of free radicals and the activation of the mitochondrial permeability transition (Obata, 2002; Jenner, 2003). The MPP^+-induced apoptosis in neuronal cells is mediated by loss of the mitochondrial transmembrane potential and results in the release of mitochondrial cytochrome *c* and subsequent activation of caspase-3 (Cassarino et al., 1999; Lotharius et al., 1999; Lee et al., 2002). In agreement with these reports, in this study, PC12 cells exposed to MPP^+ exhibit a mitochondrial dysfunction and result in the activation of caspase-3. During the apoptotic process, drops in GSH levels and concomitant increase in reactive oxygen species are detected (Tan et al., 1998; Chandra et al., 2000). The mitochondrial membrane permeability transition could induce formation of reactive oxygen species and nitrogen species by inhibition of respiratory chain (Brown, 1999; Fleury et al., 2002; Polster and Fiskum, 2004). The formation of reactive oxygen species in PC12 cells exposed to MPP^+ and the inhibitory effect of antioxidants, including Mn-TBAP and carboxy-PTIO, suggest that MPP^+ induces the formation of reactive oxygen species and nitrogen species in PC12 cells, and the mitochondrial dysfunction due to MPP^+ is mediated by oxidative stress.

Modulation of surface and mitochondrial K_{ATP} channels seems to provide a neuroprotective effect against chemical or ischemic insults (Szewczyk and Marbán, 1999; Busija et al., 2004). Antidiabetic sulfonylureas have an inhibitory effect on K_{ATP} channels in cells. Gliclazide has been suggested to reduce hydrogen peroxide-induced cell death in mouse MIN6 β-cells by a scavenging action on reactive oxygen species (Kimoto et al., 2003). However, it has been shown that glibenclamide and tolbutamide cause apoptotic cell death in cultured human pancreatic islet cells by closure of K_{ATP} channels (Maedler et al., 2005) and in rat insulinoma cell line by enhancing influx of Ca^{2+} (Iwakura et al., 2000). In addition to this controversy, the effect of glibenclamide on toxic neuronal cell injury has not been elucidated. The aim of the present study was therefore to assess whether glibenclamide modulate the mitochondrial dysfunction and cell viability loss due to MPP^+ in neuronal cells. Glibenclamide and tolbutamide reduced the cytotoxicity of MPP^+ with a maximal inhibitory at 5–10 μM; beyond these concentrations, the inhibitory effect declined. The cytotoxic effect at 20 μM supports results of the previous reports. Therefore, the toxic effect of sulfonylureas themselves at the concentrations greater than 20 μM seems to decrease or nullify the protective effect. With respect to the mitochondrial membrane permeability, the present results suggest that glibenclamide reduces the MPP^+-induced cell death in differentiated PC12 cells by suppressing the loss of mitochondrial transmembrane potential, cytochrome *c* release and subsequent caspase-3 activation. Depletion of mitochondrial GSH is demonstrated to increase the formation of reactive oxygen species, and the oxidation and depletion of GSH induce formation of the mitochondrial membrane permeability transition in rats (Constantini et al., 1996; Chandra et al., 2000). In this study, the inhibitory effect of glibenclamide on the MPP^+-induced cell death approximately correlated with the effect on GSH depletion. This finding was also observed in cells treated with tolbutamide. Therefore, the depressant effect of glibenclamide on the MPP^+-induced changes in the mitochondrial membrane permeability may be accomplished by its inhibitory action on the enhancement of reactive oxygen species formation and depletion of cellular GSH.

Overall, the results show that treatment with glibenclamide attenuates the cytotoxicity of MPP^+ in PC12 cells. Glibenclamide may reduce the MPP^+-induced viability loss in PC12 cells by suppressing changes in the mitochondrial membrane permeability, leading to the activation of caspase-3, which is associated with the increase in formation of reactive oxygen species and the depletion of GSH.

References

Brown, G.C., 1999. Nitric oxide and mitochondrial respiration. Biochim. Biophys. Acta 1411, 351–369.

Busija, D.W., Lacza, Z., Rajapakse, N., Shimizu, K., Kis, B., Bari, F., Domoki, F., Horiguchi, T., 2004. Targeting mitochondrial ATP-sensitive potassium channels—a novel approach to neuroprotection. Brain Res. Rev. 46, 282–294.

Cassarino, D.S., Parks, J.K., Parker Jr., W.D., Bennett Jr., J.P., 1999. The Parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome *c* in isolated mitochondria via an oxidative mechanism. Biochim. Biophys. Acta 1453, 49–62.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323–333.

Constantini, P.C., Chernyak, B.C., Petronilli, V., Bernardi, P., 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J. Biochem. Biol. 271, 6746–6751.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233–249.

Emilien, G., Maloteaux, J.M., Ponchon, M., 1999. Pharmacological management of diabetes: recent progress and future perspective in daily drug treatment. Pharmacol. Ther. 81, 37–51.

Fleury, C., Mignotte, B., Vayssiere, J.-L., 2002. Mitochondrial reactive oxygen species in cell death signaling. Biochimie 84, 131–141.

Fonck, C., Baudry, M., 2001. Toxic effects of MPP^+ and MPTP in PC12 cells independent of reactive oxygen species formation. Brain Res. 905, 199–206.

Fu, W., Luo, H., Parthasarathy, S., Mattson, M.P., 1998. Catecholamines potentiate amyloid β-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. Neurobiol. Dis. 5, 229–243.

Goodman, Y., Mattson, M.P., 1996. K^+ channel openers protect hippocampal neurons against oxidative injury and amyloid β-peptide toxicity. Brain Res. 706, 328–332.

Isenberg, J.S., Klaunig, J.E., 2000. Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. Toxicol. Sci. 53, 340–351.

Iwakura, T., Fujimoto, S., Kagimoto, S., Inada, A., Kubota, A., Someya, Y., Ihara, Y., Yamada, Y., Seino, Y., 2000. Sustained enhancement of Ca^{2+} influx by glibenclamide induces apoptosis in RINm5F cells. Biochem. Biophys. Res. Commun. 271, 422–428.

Jenner, P., 2003. Oxidative stress in Parkinson's disease. Ann. Neurol. 53 (suppl 3), S26–S38.

Kadota, T., Yamaai, T., Saito, Y., Akita, Y., Kawashima, S., Moroi, K., Inagaki, N., Kadota, K., 1996. Expression of dopamine transporter at the tips of growing neurites of PC12 cells. J. Histochem. Cytochem. 44, 989–996.

Kimoto, K., Suzuki, K., Kizaki, T., Hitomi, Y., Ishida, H., Katsuta, H., Itoh, E., Ookawara, T., Suzuki, K., Honke, K., Ohno, H., 2003. Gliclazide protects pancreatic β-cells from damage by hydrogen peroxide. Biochem. Biophys. Res. Commun. 303, 112–119.

Lee, C.S., Han, E.S., Jang, Y.Y., Han, J.H., Ha, H.W., Kim, D.E., 2000. Protective effect of harmalol and harmaline on MPTP neurotoxicity in the mouse and dopamine-induced damage of brain mitochondria and PC12 cells. J. Neurochem. 75, 521–531.

Lee, C.S., Han, J.H., Jang, Y.Y., Song, J.H., Han, E.S., 2002. Differential effect of catecholamines and MPP^+ on membrane permeability in brain mitochondria and cell viability in PC12 cells. Neurochem. Int. 40, 361–369.

Lotharius, J., Dugan, L.L., O'Malley, K.L., 1999. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J. Neurosci. 19, 1284–1293.

Maedler, K., Carr, R.D., Bosco, D., Zuellig, R.A., Berney, T., Donath, M.Y., 2005. Sulfonylurea induced β-cell apoptosis in cultured human islets. J. Clin. Endocrinol. Metab. 90, 501–516.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nagy, K., Kis, B., Rajapakse, N.C., Bari, F., Busija, D.W., 2004. Diazoxide preconditioning protects against neuronal cell death by attenuation of oxidative stress upon glutamate stimulation. J. Neurosci. Res. 76, 697–704.

Obata, T., 2002. Dopamine efflux by MPTP and hydroxyl radical generation. J. Neural. Transm. 109, 1159–1180.

Obata, T., 2003. Calcium overload enhances hydroxyl radical generation by 1-methyl-4-phenylpyridinium ion (MPP^+) in rat striatum. Brain Res. 965, 287–289.

Oberhammer, F.A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A.F., Bursch, W., Schulte-Hermann, R., 1992. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc. Natl. Acad. Sci. U. S. A. 89, 5408–5412.

Polster, B.M., Fiskum, G., 2004. Mitochondrial mechanisms of neural cell apoptosis. J. Neurochem. 90, 1281–1289.

Rojas, P., Rios, C., 1993. Increased striatal lipid peroxidation after intracerebroventricular MPP^+ administration to mice. Pharmacol. Toxicol. 72, 364–368.

Rustenbeck, I., Krautheim, A., Jorns, A., Steinfelder, H.J., 2004. β-Cell toxicity of ATP-sensitive K^+ channel-blocking insulin secretagogues. Biochem. Pharmacol. 67, 1733–1741.

Szewczyk, A., Marbán, E., 1999. Mitochondria: a new target for K^+ channel openers? Trends Pharmacol. Sci. 20, 157–161.

Tan, S., Sagara, Y., Liu, Y., Maher, P., Schubert, D., 1998. The regulation of reactive oxygen species production during programmed cell death. J. Cell Biol. 141, 1423–1432.

Tatton, W.G., Chalmers-Redman, R.M.E., Ju, W.J.H., Mammen, M., Carlile, G. W., Pong, A.W., Tatton, N.A., 2002. Propargylamines induce antiapoptotic new protein synthesis in serum-and nerve growth factor (NGF)-withdrawn, NGF-differentiated PC-12 cells. J. Pharmacol. Exp. Ther. 301, 753–764.

van Klaveren, R.J., Hoet, P.H.M., Pype, J.L., Demedts, M., Nemery, B., 1997. Increase in γ-glutamyltransferase by glutathione depletion in rat type II pneumocytes. Free Radic. Biol. Med. 22, 525–534.